

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:  
Brockhaus et al.

Application No. 08/444,790

Art Unit: 1646

Filed May 19, 1995

Examiner: Z. Howard

For: HUMAN TNF RECEPTOR

**SECOND DECLARATION OF STEWART LYMAN, Ph.D.,  
UNDER 37 C.F.R. § 1.132**

I, Stewart Lyman, Ph.D., declare as follows that:

1. I have been informed that the applicants of the above-identified application seek priority benefit of an earlier application, European application No. 90116707.2 filed August 31, 1990 ("August 31, 1990 Priority Application"). I have read what I have been informed is an English translation of this application, attached hereto as Appendix B.
2. I have considered the contents of the English translation of the August 31, 1990 Priority Application, the knowledge and understanding of a person skilled in the art as of August 31, 1990, and the statements I made in my prior declaration entitled "Declaration of Stewart Lyman, Ph.D. under 37 C.F.R. 1.132" regarding the above-identified application and the Office Action dated February 23, 2007.
3. The portions of the above-identified application that I referenced in my prior declaration also appear in the English translation of the August 31, 1990 Priority Application, including the citation to the Smith (1990) Science article. The table attached as Appendix A shows the page and line numbers of the corresponding cited passages in the English translation of the August 31, 1990 Priority Application.
4. All of the statements that I made in my earlier declaration with respect to what a person skilled in the art would understand from reading the text of the above-identified application as of its September 10, 1990 effective filing date apply equally to what a person skilled in the art would understand from reading the English

translation of the August 31, 1990 Priority Application as of its filing date. In other words, the written description conveyed by the above-identified application as of September 10, 1990 is the same as the written description conveyed by the English translation of the August 31, 1990 Priority Application as of its filing date.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

July 17, 2007  
Date

Stewart Lyman  
Dr. Stewart Lyman

**APPENDIX A**

Location of passage in U.S. Appl. No. 08/444,790	Location of passage in the English translation of the August 31, 1990 Priority Application
Figure 1, pages 1 and 2 of the drawings	Figure 1, pages 21 and 22
Figure 4, pages 5 and 6 of the drawings	Figure 4, pages 25 and 26
Page 3, lines 14-16	Page 3, lines 12-14
Page 3, line 35-page 4, line 3	Page 4, lines 11-16
Page 7, lines 13-16	Page 2b, lines 1-3
Page 9, line 19-page 10, line 10	Page 3a, line 13-page 3b, line 1
Page 10, lines 9-10 (citation to Smith (1990) article)	Page 3b, line 1 (citation to Smith (1990) article)
Page 10, line 19-23	Page 3b, lines 7-9
Page 10, lines 23-26	Page 3b, lines 9-11
Page 14, lines 32-36	Page 5, line 27-page 5a, line 2
Page 17, lines 18-31	Page 6, lines 20-28
Page 20, lines 27-30	Page 7, lines 24-26
Page 33, lines 7-19	Page 13a, line 23-page 14, line 1
Page 35, lines 22-33	Page 14a, lines 20-28
Page 37, lines 14-18	Page 15a, lines 8-11
Page 42, lines 5-8	Page 17a, lines 9-11
Page 42, lines 5-25 (Example 11)	Page 17a, lines 9-21 (Example 11)
Example 11, page 42, line 1-page 43, line 8	Example 11, page 17a, line 9-page 18, line 9



APPENDIX B

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54 TNF-binding Proteins

57 The present invention relates to non-soluble proteins as well as to their soluble and non-soluble fragments, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially such proteins with a molecular mass of 55 or 75 kDa (non-reducing SDS-PAGE conditions), processes for isolation of such protein antibodies against such proteins, DNA sequences, which are coded for non-soluble proteins as well as for their soluble or non-soluble fragments, which bind TNF, as well as those encoded for proteins, consisting in one part of a soluble fragment that binds TNF and in another part of all domains save the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins thus encoded as well as processes for their production by means of transformed prokaryotic as well as eukaryotic cells.

TNF-BINDING PROTEINS

The Tumor Necrosis Factor  $\alpha$  (TNF  $\alpha$ , also cachectin), discovered because of its hemorrhagic-necrotizing effect on certain tumors, and lymphotoxin (TNF  $\beta$ ) are two closely related peptide factors [3] of the class of lymphokines/cytokines, which will both from now on be designated as TNF [ see survey papers 2 and 3 ]. TNF disposes over a broad cellular performance spectrum. For instance TNF has an inhibiting or cytotoxic effect on a series of tumor cell lines [2,3], stimulates the proliferation of fibroblasts and the phagocytizing/cytotoxic activity of myeloid cells [4,5,6], induces adhesion molecules in endothelial cells or has an inhibiting effect on endothelium [7,8,9,10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histocompatibility antigens [12]. Some of these TNF effects are achieved via induction from other factors or through synergistic effects with other factors, such as interferons or interleukins [13-16], for instance.

TNF is involved in a series of pathological conditions, for instance in shock conditions during meningococcal sepsis [17], during development of autoimmune glomerulonephritis in mice [18] or in case of cerebral malaria in mice [19] and humans [41]. Generally speaking, the toxic effects of endotoxin appear to be transmitted through TNF [20]. Furthermore, TNF can, just like interleukin-1, bring on fever [39]. Based on the pleiotropic functional characteristics of TNF it can be assumed that TNF is a participant in interaction with other cytokines in a whole series of further pathological conditions as mediator of immune response, inflammation or other processes.

~~These biological effects are transmitted through TNF via specific receptors,~~  
with TNF  $\alpha$  and TNF  $\beta$  both binding to the same receptors, according to the present

state of knowledge [21]. Different types of cells are differentiated by the number of TNF receptors [22,23,24]. Such quite generally considered TNF binding proteins (TNF-BP) were verified through covalent binding to radioactive marked TNF [24-29], with the following apparent molecular masses of the obtained TNF/TNF-BP complexes having been determined: 95/100 kDa and 75 kDa [24], 95 kDa and 75 kDa [25], 138 kDa, 90 kDa, 75 kDa, and 54 kDa [26].  $100 \pm 5$  kDa [27], 97 kDa and 70 kDa [28] and 145 kDa [29]. By means of anti-TNF-antibody-immunoaffinity chromatography and preparative SDS-polyacrilamide gel electrophoresis (SDS-PAGE) such a TNF/TNF-BP complex could be isolated [27]. The reductive cleavage of this complex and the subsequent SDS-PAGE analysis resulted in several bands, which were not tested for TNF binding activity, however. Since the specific conditions, which must be employed for cleavage of the complex, lead to an inactivation of the binding protein [31], the latter was made impossible. The enrichment of soluble TNF-BP from human serum or urine by means of ion exchange chromatography and gel filtration (molecular masses in the range of 50 kDa) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation through TNF $\alpha$ -ligandaffinity chromatography and HPLC from membrane extracts of HL60 cells, which was in turn used as antigen preparation for the production of monoclonal antibodies against TNF-BP. Through use of such an immobilized antibody (immunoaffinity chromatography) an enriched preparation of TNF-BP was obtained by means of TNF $\alpha$  ligandaffinity chromatography and HPLC by Loetscher and Brockhaus [31] from an extract of human placenta, which exhibited a strong wide band at 35 kDa, a weak band at about 40 kDa and a very weak band in the region between 55 kDa and 60 kDa, during the SDS-PAGE analysis. Moreover the gel exhibited a background smear in the region between 33 kDa and 40 kDa. The meaning of the protein bands thus obtained was not clear, however, in view of the heterogeneity of the initial material used (placenta tissue; material combined from several placentas).

The subject of the present invention is non-soluble proteins, i.e., for instance membrane proteins resp. so-called receptors and their soluble or non-soluble fragments, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred are those proteins which are characterized, according to SDS-PAGE under non-reducing conditions, by apparent molecular masses of about 55 kDa, 51 kDa, 38 kDa, 36 kDa, 36 kDa and 34 kDa resp. 75 kDa and 65 kDa particularly those with about 55 kDa and 75 kDa. Additionally preferred are those proteins, which are characterized by at least one of the following amino-acid part sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu



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(IIB) Val-Phe-Cys-Thr

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala

(IID) Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu

(IIF) Leu-Cys-Ala-Pro

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro where X stands for an amino acid residue that could not be determined unequivocally.

In the state of the technology TNF-BP have already been characterized by an N-terminal partial sequence [European Patent Application Nr. 308 378], with this sequence differing from the N-terminal partial sequence according to the invention according to formula (IA). The TNF binding proteins described in the state of the technology relate by the way to soluble i.e. not membrane-bound TNF-BP isolated from urine and not to membrane-bound, i.e. non-soluble, TNF-BP.

Also subject of the present application are processes for isolation of the TNF-BP according to the invention. These processes are characterized in that basically the following steps of purification are being carried out sequentially: production of a cell extract or tissue extract, immunoaffinity chromatography and/or simple or multiple ligandaffinity chromatography, high pressure liquid chromatography (HPLC) and preparative SDS-polyacrilamide gel electrophoresis (SDS-PAGE). The combination of the individual purification steps known from the state of current technology is essential for the success of the process according to the invention, with individual steps within the scope of the task to be solved having been modified and improved. For instance the combined immunoaffinity chromatography/TNF $\alpha$ -ligandaffinity chromatography step, initially used for enrichment of TNF-BP through human placenta [31], was changed in that a BSA-Sepharose 4B - precolumn was used. This precolumn was put in series with the immunoaffinity column and followed by the ligandaffinity column for application of the cell

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extract or membrane extract. After application of the extract the two columns mentioned last were decoupled, each eluated separately and the TNF-BP active fractions were purified once more through a ligandaffinity column. Essential for the carrying out of the reverse phase HPLC step according to the invention is the use of a solvent mixture containing a detergent.

Furthermore a technical process to obtain high cell densities of mammalian cells, from which TNF-BP can be isolated, is also a subject of the present invention. Such a process is characterized in that a medium, which was developed for the specific growth requirements of the cell line used, was employed in combination with a perfusion instrument as described in detail in Example 2, for instance. By means of such a process cell densities for HL-60 cells may be increased to as much as 20 times higher than usual.

In addition the present invention relates to DNA sequences, which are coded for proteins that bind TNF and for their soluble and non-soluble fragments. By that we understand for instance DNA sequences that are coded for non-soluble proteins that bind TNF, or for their soluble and non-soluble fragments, with such DNA sequences being selectable from the following:

- (a) DNA sequences, as shown in Fig.1 or Fig.4, as well as their complete strands, or those that include these sequences;
- (b) DNA sequences that hybridize with sequences defined in (a) or with their fragments;
- (c) DNA sequences that do not hybridize with sequences as defined in (a) and (b) because of degeneration of the genetic code, but which code for polypeptides with exactly the same amino-acid sequence.

That means that the present invention includes not only allelic variants but also such DNA sequences that result from deletions, substitutions and additions of one or more nucleotides of the sequences shown in Fig.1 resp. Fig.4, with the thus coded proteins being at all times TNF-BP. A sequence resulting from such a

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deletion is described in SCIENCE 248, 1019-1023, (1990), for instance.

Preferred are such DNA sequences that are coded for such a protein with an apparent molecular mass of about 55 kDa, with the sequence shown in Fig.1 being especially preferred as are sequences that are coded for non-soluble or soluble fragments of such proteins. A DNA sequence that is, for instance, coded for such a non-soluble protein fragment stretches from nucleotide -185 to 1122 of the sequence shown in Fig.1. DNA sequences that are coded for soluble protein fragments are, for instance, those that stretch from nucleotide -185 to 633 resp. from nucleotide -14 to 633 of the sequence shown in Fig.1. Also preferred are DNA sequences that are coded for a protein of about 75/65 kDa, with preference for those containing the partial cDNA sequences shown in Fig.4.

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Especially preferred DNA sequences in this case are the sequences of the open reading frame from nucleotide 2 to 1177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are encoded by the partial cDNA sequence in Fig.4, where the slight deviations in the experimentally determined amino acid sequences from the sequence derived from cDNA are most probably caused by the smaller resolution of gas phase sequencing. Also preferred are DNA sequences, which are coded for non-soluble as well as soluble fragments of TNF binding proteins with an apparent molecular mass of 75 kDa/65 kDa. DNA sequences for such soluble fragments can be determined based on the hydrophilia profiles of the amino sequences derived from nucleic acids coded for such non-soluble TNF-BP.

The invention relates furthermore to DNA sequences, which comprise a combination of two partial DNA sequences, with one partial sequence being coded for such soluble fragments of non-soluble proteins which bind TNF (above) while the other partial sequence is coded for all the domains, save the first domain of the constant region of the heavy chain of human immunoglobulins, like IgG, IgA, IgM resp. IgE.

The present invention relates naturally also to recombinant proteins encoded by such DNA sequences. It is self-evident that such proteins, in whose amino acid sequences amino acids have been exchanged, for instance by means of directed mutagenesis, in such a way that the activity of the TNF-BP or their fragments, specifically the binding of TNF or the interaction with other membrane components involved in the signal transmission, were changed or retained in a desired manner, are also included. Amino acid exchanges in proteins and peptides, which in general do not change the activity of such molecules, are known in the current state of technology and have been described, for instance, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York 1979, see in particular Fig.6, page 14). The exchanges occurring most frequently are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala, Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn.

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Leu/Ile, Leu Val, Ala/Glu, Asp/Gly, as well as in reverse direction. The present invention relates further to vectors that contain DNA sequences according to the invention and are suitable for the transformation of suitable prokaryotic as well as eukaryotic host systems, with such vectors being preferred whose use leads to the expression of the proteins encoded by the DNA sequences according to the invention. Finally the invention relates also to prokaryotic as well as eukaryotic host systems transformed with such vectors, such as processes for production of recombinant combinations through cultivation of such host systems and subsequent isolation of these combinations from the host systems themselves or from their culture supernatants.

Also subject of the present invention are pharmaceutical preparations, which contain at least one of these TNF-BP or their fragments, if so desired in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

The present invention relates finally to the use of such TNF-BP on the one hand for production of pharmaceutical preparations, resp. on the other hand to the treatment of diseases, preferably those in the course of which TNF is involved.

The starting material for the TNF-BP according to the invention are in general cells that contain such TNF-BP in membrane-bound form and which are generally available to the specialist without any restrictions, like for instance HL60- [ ATCC Nr. CCL 240 ], U 937- [ ATCC Nr. CRL 1593 ], SW 480- [ ATCC Nr. CCL 228 ] and HEp2 cells [ ATCC Nr. CCL 23 ]. These cells may be cultivated according to known methods of the current state of technology [40] or for the attainment of high cell densities by means of the already in general and in detail described process for HL60 cells in example 2. TNF-BP may then be extracted according to known methods of the current state of technology by means of suitable detergents, for instance Triton X-114, 1-O-n-octyl- $\beta$ -D-glucopyranoside (octylglucoside), or 3-[( 3-cholylamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), particularly

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by means of Triton X-100, from the cells that have been centrifuged off the medium and washed. The usually employed methods of identification for TNF-BP, for instance a polyethylene glycol-induced precipitation of the  $^{125}\text{I}$ -TNF/TNF-BP complex [27], in particular filter binding tests with radioactively marked TNF according to Example 1, may be used. The current state of technology methods generally employed for purification of proteins, particularly of membrane proteins, such as ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE, may be used for the recovery of the TNF-BP according to the invention. Particularly preferred methods for the production of TNF-BP according to the invention are affinity chromatography, particularly with TNF- $\alpha$  as the ligand tied to the solid phase and immunoaffinity chromatography, HPL and SDS-PAGE. The elution of TNF-BP bands separated by means of SDS-PAGE can occur according to known methods of protein chemistry, for instance by means of electro-elution according to Hunkapiller et al. [34], with the electrodialysis times listed there to be doubled in general according to the present state of knowledge. Still remaining traces of SDS may then be removed according to Bosserhoff et al. [50].

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Having been thus purified the TNF-BP may be characterized by means of the methods of peptide chemistry known at the current state of technology, as for instance N-terminal amino acid sequencing or enzymatic as well as chemical peptide cleavage. Fragments obtained through enzymatic or chemical cleavage may be separated by familiar methods, like HPLC for instance and then be subject of N-terminal sequencing themselves. Such fragments, which still bind TNF, may be identified by means of the methods of identification for TNF-BP listed above and are also a subject of the present invention.

Starting with the information about amino acid sequences or about the DNA sequences as well as amino acid sequences shown in Fig.1 and Fig.4 thus obtainable suitable oligonucleotides may be produced with methods known at the current state of technology while observing the genetic code degeneration [51]. With them and by again using well known methods of molecular biology [42,43] cDNA banks or genomic DNA banks may be scanned for clones containing nucleic acid sequences encoded for TNF-BP. In addition cDNA fragments may be cloned by means of the polymerase chain reaction (PCR)[49] by inserting completely degenerated and due to their complementarity suitable oligonucleotides that come from separate, relatively short sections of the amino acid sequence and by observing the genetic code, as "primers", which enables amplification and identification of the fragment that lies between these two sequences. Determination of of the nucleotide sequence of such a fragment makes possible an independent determination of the amino acid sequence of the protein fragment for which it is encoded. The cDNA fragments obtainable through PCR may also be used, as already described for the oligonucleotides proper, by means of known methods, for the search for clones encoded for TNF-BP and containing nucleic acid sequences from cDNA banks resp. genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. Based on the sequences thus determined as well as on those already known for certain receptors, such partial sequences which are encoded for soluble TNF-BP

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fragments, may be determined and cut out from the total sequence by means of known methods [42].

The total sequence or such partial sequences may then be integrated by means of known methods into vectors described in the current state of technology for their multiplication and as well as expression in prokaryotes [42]. Suitable prokaryotic host organisms are for instance gram-negative as well as gram-positive bacteria, like for instance *E. coli* strains, like *E. coli* HB 101 [ATCC Nr. 33 694] or *E. coli* W3110 [ATCC Nr. 27 325] or *B. subtilis* strains.

Furthermore nucleic acid sequences according to the invention, which are encoded for TNF-BP as well as for TNF-BP fragments, may be integrated into suitable vectors for proliferation as well as expression in eukaryotic host cells, like for instance yeast, insect cells and mammal cells, by means of known methods. Expression of such sequences occurs preferably in mammal as well as in insect cells.

A typical expression vector for mammal cells contains an efficient promoter element, for achievement of a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements that can be used are "enhancers", which lead to further amplified transcription and sequences, which may for instance effect a longer biological half-life for the mRNA. For the expression of nucleic acid sequences in which the endogene sequence piece encoded for a signal peptide is missing, vectors may be used that contain such suitable sequences encoded for signal peptides of other known peptides. See for instance the vector pLJ268 described by Cullen, B.R. in Cell 46, 973-982(1986) or also by Sharms, S. et al. in "Current Communications in Molecular Biology", ed. by Gething, M.J. Cold Spring Harbor Lab.(1985), pages 73-78.

Most of the vectors used for a transient expression of a specific DNA sequence ~~in mammal cells contain the replication origin of the SV40 virus. In cells that express the T-antigen of the virus, (for instance COS cells), these vectors pro-~~



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liferate strongly. But a temporary expression is not limited to COS cells. In principle any mammal cell may be used for that purpose. Signals that can effect a strong transcription are, for instance, the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses, like for instance RSV, HIV and MMTV. But signals from cellular genes, like for instance the promoters of the actin genes and collagenase genes, can be used.

Alternatively, stable cell lines that have integrated the specific DNA sequence in the genome (chromosome), may be obtained. For that the DNA sequence is co-transfected together with a selectable marker, for instance neomycin, hygromycin, dihydrofolate-reductase (dhfr) or hypoxanthine-guanine-phosphoribosyltransferase (hgbt).

The DNA sequence which is stably built into the chromosome can also be considerably increased. A suitable selection marker for it is the dihydrofolate-reductase (dhfr) for example. Mammal cells ( for instance CHO cells ), which contain no intact dhfr gene, will in that case be incubated with increasing amounts of methotrexate after completed transfection. In that way cell lines can be preserved that contain more than a thousand copies of the desired DNA sequence.

Mammal cells, which can be used for the expression, are for example cells of the human cell lines HeLa [ATCC CCL2] and 293 [ATCC CRL 1573], as well as 3T3- [ATCC CCL 163] and L cells, for instance [ATCC CCL 149], (CHO) cells [ATCC CCL 61], BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] cell lines and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include for example vectors like pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVCat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. Particularly preferred vectors are the vectors "pK19" and "pN123" used in Example 9. These can be isolated from the E. coli strains HB101(pK19) and HB101(pN123), which are transformed with them, by means of known methods [42]. These E. coli strains were deposited on 26. January 1990 with the German Collection of Micro-Organisms and Cell Cultures, Inc. (DSM) in Braunschweig (BRD) [form. West Germany ..Transl..] under DSM 5761 for HB101(pK19) and DMS 5764 for HB101(pN123). For the expression of proteins, which consist of a soluble fragment from non-soluble TNF-BP and an immunoglobulin component, i.e. all domains but the first from the constant region of the heavy chain, vectors derived from pSV2 like for example described by German, C. in "DNA Cloning" [Vol. II. ed. by Glover, D.M., IRL Press Oxford, 1985], are particularly suitable. Particularly preferred vectors are the vectors pCD4-Hu (DSM 5315), pCD4-H 1 (DSM 5314) and pCD4-H 3 (DSM 5523), which are deposited with the German Collection of Micro-Organisms and Cell Cultures, Inc. (DSM) in Braunschweig, BRD and described in detail in the European Patent Application Nr. 90107393.2. The said European patent

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specification mentioned, as well as the equivalent patent applications listed in Example 11, also contain material relating to the further use of these vectors for the expression of such chimeric proteins with other immunoglobulin components.

The nature and manner in which the cells are being transfected depends on the expression system and vector system chosen. A survey of these methods is found for example at Pollard et al. , "DNA Transformation of Mammalian Cells" in "Methods of Molecular Biology" [Nucleic Acids Vol.2, 1984, Walker, J.M., ed, Humana, Clifton, New Jersey]. Further methods are found at Chen and Okayama [" High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and at Felgner [Felgner et al., "Lipofectin: a highly efficient lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

For the expression in insect cells the Baculovirus-Expression-System, which has already been employed successfully for the expression of a series of proteins, (for a summary see Luckow and Summers, Bio/Technology 6, 47-55, 1988), can be used. Recombinant proteins can be produced authentically or as fusion proteins. The proteins thus produced may also be modified, for example be glycosylized (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). To produce a recombinant Baculovirus, which expresses the desired protein, a so-called "transfer vector " is employed. This is understood to be a plasmid, which contains the heterologous DNA sequence under control of a strong promoter, for instance that of the polyhedrin gene, with the sequence being surrounded on both sides by viral sequences. Particularly preferred vectors are "pN113", "pN119" and "pN124", the vectors used in Example 10. These can be isolated according to known methods [42] from the E. coli strains HB101(pN113), HB101(pN119) and HB101(pN124). These E. coli strains were deposited with the German Collection of Micro-Organisms and Cell Cultures, Inc.

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(DSM) in Braunschweig, BRD, under DSM 5762 for HB101(pN113), DSM 5763 for HB101(pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected together with DNA from the wild type Baculovirus into the insect cells. The recombinant viruses that are generated in the cells through homologous recombination may then be identified and isolated according to known methods. A survey about the Baculovirus-Expression-System is found at Luckow and Summers [52].

Expressed TNF-BP as well as their non-soluble and soluble fragments can then be purified out through methods of the protein chemistry known at the current state of the technology, as for example the methods already described on pages 5-6, from the cell mass and or the culture supernatants.

The TNF-BP recovered according to the invention may also be used for the production of polyclonal and monoclonal antibodies according to known methods of the technology [44,45] or according to the method described in Example 3.

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Such antibodies, particularly monoclonal antibodies against the 75 kDa TNF-BP species, are also a subject of the present invention. Such antibodies directed against the 75 kDa TNF-BP can be employed for isolation of TNF-BP by means of modifications familiar to the specialist to the purification method described in detail in Examples 4-6.

Based on the high binding affinity for TNF of the TNF-BP according to the invention ( $K_d$  values of the orders of magnitude of  $10^{-9}$  -  $10^{-10}$  M) they or fragments thereof may be used as diagnostics for identification of TNF in serum or in other body fluids according to methods known at the current state of technology, for instance in solid phase binding tests or in combination with anti-TNF-BP antibodies on so-called "sandwich" tests.

In general TNF-BP may be used according to the invention for purification of TNF on the one hand and for the location of TNF-agonists as well as TNF-antagonists by means of methods known at the current state of technology on the other.

The TNF-BP according to the invention as well as their physiologically compatible salts, which can be produced according to the methods known at the current state of technology, may also be used for production of pharmaceutical preparations in particular those for treatment of diseases in the course of which TNF is involved. One or several of the mentioned compounds, if desired resp. required in combination with other pharmaceutically active substances, may be processed in a known manner with the usually employed solid or liquid carrier materials. The dosage of such preparations can occur with reference to the usual criteria in analogy to preparations of similar activity and structure already in use.

After having described the above invention in general, the following examples are to illustrate details of the invention without limiting it by that in any way.

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Example 1Detection of TNF-binding Proteins

The TNF-BP were detected in a filter test with human radioactive iodine  $^{125}\text{I}$ -TNF. TNF (46,47) was marked radiactively with  $\text{Na}^{125}\text{I}$  (IMS40, Amersham, Amersham, England) and Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. Isolated membranes of the cells or their solubilized, enriched and purified fractions, were applied to wetted cellulose nitrate filters (0.45  $\mu$ , Bio.Rad, Richmond, CA, USA) for detection of the TNF-BP. The filter was then blocked in buffer solution with 1% of defatted powdered milk and subsequently incubated, washed and airdried with  $5 \cdot 10^5$  cpm/ml  $^{125}\text{I}$ -TNF $\alpha$  ( $0.3\text{--}1.0 \cdot 10^8$  cpm/ $\mu\text{g}$ ) in two batches with and without addition of 5  $\mu\text{g}/\text{ml}$  of unmarked TNF $\alpha$ . The bound radioactivity was detected through autoradiography semiquantitatively or counted in a  $\gamma$ -counter. The specific  $^{125}\text{I}$ -TNF $\alpha$  binding was obtained after correction for nonspecific binding in the presence of unmarked TNF- $\alpha$  in the surplus. The specific TNF binding during the filter test was measured at various TNF concentrations and analyzed according to Scatchard [33], with a  $K_d$  value of  $\sim 10^{-9}$  -  $1 \cdot 10^{-10}$  having been obtained.

Example 2Cell Extracts from HL-60 Cells

HL60 cells [ATCC Nr. CCL 240] were cultivated on an experimental laboratory scale in an RPMI 1640-Medium [GIBCO catalog Nr. 074-01800], that also contained 2 g/l  $\text{NaHCO}_3$  and 5% total calves' serum, within an atmosphere of 5%  $\text{CO}_2$  and were subsequently centrifuged.

The following procedure was used to obtain high cell densities on a technical scale. Breeding was carried out in a 75 liter airlift fermenter (Chemap. Co. Swi-

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tzerland) with 58 liter of working volume. For that purpose the cassette membrane system "PROSTAK" (Millipore, Switzerland) with a membrane surface of  $0.32 \text{ m}^2$  (1 cassette) was integrated into the external circulation. The culture medium (see Table 1) was recirculated at 5 l/min. with a Watson-Marlow pump Type 603U. After sterilization of the equipment with steam, with the "PROSTAK" system being separately autoclaved, fermentation was started with growing HL-60 cells from a 20 l airlift fermenter (Chemap). Cell breeding in the seed fermenter was carried out in the medium through a conventional batch process according to Table 1 and an initial cell titre of  $2 \times 10^5$  cells/ml. After 4 days the HL60 batch with a titre of  $4.9 \times 10^6$  cells/ml was transferred to the 75 l fermenter. The pH value was kept at 7.1 and the  $pO_2$  value at 25% saturation, with oxygen being fed through a micro-porous frit. After initial batch formation the perfusion was started on the second day with a cell titre of  $4 \times 10^6$  cells/ml at a medium exchange rate of 30 l per day. On the filtrate side of the membrane the conditioned medium was withdrawn and replaced by inflow of fresh medium. The inflowing medium was strengthened as follows: primatone from 0.25% to 0.35%, glutamin from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then raised on the third and fourth day to 72 l of medium/day and on the fifth day to 100 l of medium/day. After 120 hours of continuous breeding the fermentation was finished. Exponential cell growth up to  $40 \times 10^6$  cells/ml occurred under the given conditions of fermentation. The period for doubling the cell population was 20-22 hours up to  $10 \times 10^6$  cells/ml and increased to 30-36 hours with increasing cell density. The share of the living cells was around 90-95% during the entire period of fermentation. The HL-60 batch was then cooled down to about  $12^\circ\text{C}$  in the fermenter and the cells were recovered through centrifugation (Beckmann centrifuge [model J-6B, rotor JS], 3000 rpm, 10 min.,  $4^\circ\text{C}$ ).

Table 1HL -60 Medium

Components	Concentrations mg/l
$\text{CaCl}_2$ ( moisture free)	112,644
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	.20
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$0.498 \cdot 10^{-3}$
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	0.02
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1668
KCl	336.72
$\text{KNO}_3$	0.0309
$\text{MgCl}_2$ (moisture free)	11.444
$\text{MgSO}_4$ (moisture free)	68.37
NaCl	5801.8
$\text{Na}_2\text{HPO}_4$ (moisture free)	188,408
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	75
$\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$	$9.6 \cdot 10^{-3}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1726
D-Glucose	4000
Glutathion ( red.)	0.2
Hepes-Buffer	2383.2
Hypoxanthin	0.954
Linoleic Acid	0.0168
Liponic Acid	0.042
Phenol Red	10.24
Putrescin 2HCl	0.0322
Na-Pyruvat	88
Thymidin	0.146



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Biotin	0.04666
D-Ca-Pantothenate	2.546
Choline Chloride	5.792
Folic Acid	2.86
i-Inositol	11.32
Niacin Amide	2.6
para- Amino-Benzoic Acid	0.2
Pyridoxal HCL	2.4124
Pyridoxin HCL	0.2
Riboflavin	0.2876
Thiamin HCL	2.668
Vitamin B <sub>12</sub>	0.2782
L- Alanine	11.78
L- Asparagine Acid	10
L- Asparagine H <sub>2</sub> O	14.362
L- Arginine	40
L- Arginine HCL	92.6
L- Aspartate	33.32
L- Cystine 2HCL	62.04
L- Cysteine HCL •H <sub>2</sub> O	7.024
L- Glutamine Acid	36.94
L- Glutamine	730
L- Glycine	21.5
L- Histidine	3
L- Histidine HCL •H <sub>2</sub> O	27.392
L- Hydroxypyroline	4
L- Isoleucine	73.788
L- Leucine	75.62

L- Lysine HCl	102.9
L- Methionine	21.896
L- Phenylalanine	43.592
L- Proline	26.9
L- Serine	31.3
L- Threonine	53
L- Tryptophan	11.008
L- Tyrosine •2Na	69.76
L- Valine	62.74
Penicillin/Streptomycin	100 U/ml
Insulin (human)	5 µg/ml
Transferrin (human)	15 µg/ml
Bovine Serum Albumin	67 µg/ml
Primatone RL (Sheffield Products, Norwich, N.Y., USA)	0.25%
Pluronic F68 (Serva, Heidelberg, BRD)	0.01%
Fetal Calves' Serum	0.3-3%

The centrifugate was washed with isotonic phosphate buffer (PBS; 0,2g/l KCl, 0.2 g/l  $\text{KH}_2\text{PO}_4$ , 8.0 g/l NaCl, 2.16 g/l  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ), to which were added 5% dimethyl formamide, 10 mM benzamidine, 100 E/ml aprotinine, 10 µM leupeptine, 1 µM pepstatine, 1 mM o-pheanthroline, 5 mM iodacetamide, 1mM phenylmethylsulphonyl fluoride (and which will be referred to as PBS-M from now on). The washed cells were extracted at a cell density of  $2.5 \cdot 10^8$  cells/ml in PBS-M with Triton X-100 ( final concentration 1.0%). The cell extract was clarified through centrifugation (  $15'000 \times g$ , 1 hour;  $100'000 \times g$ , 1 hour).

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Example 3Production of monoclonal (TNF-BP) Antibodies

A supernatant of centrifugation obtained according to Example 2 from cultivation of HL60 cells on an experimental laboratory scale was diluted 1:10 with PBS. The diluted supernatant was applied at 4°C to a column (flow rate: 0.2 ml/min), which contained 2 ml Affigel 10 (Bio Rad catalog Nr. 153-6099), to which 20 mg of recombinant human TNF- $\alpha$  [Pennica, D. et al. (1984) *Nature* 312, 724; Shirai, T. et al. (1985) *Nature* 313, 803; Wang, A.M. et al. (1985) *Science* 228, 149] were coupled according to the suggestions of the manufacturer. The column was washed at 4°C and a flow-through rate of 1 ml/min first with 20 ml of PBS containing 0.1% Triton X 114 and then with 20 ml PBS. The thus enriched TNF-BP was eluted at 22°C and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decylmaltoside. The eluate was concentrated to 10  $\mu$ l in a Centricon 30 unit [Amicon].

10  $\mu$ l of this eluate were mixed with 20  $\mu$ l of complete Freund's adjuvant to an emulsion. According to the procedure described by Holmdahl, R. et al. [(1985), *J. Immunol. Methods* 83 379] 10  $\mu$ l of the emulsion were injected on days 0, 7 and 12 into a rear paw of an anesthetized Balb/c mouse.

On day 14 the immunized mouse was killed, the popliteal lymph node removed, chopped up and suspended in Iscove's medium (MEM, GIBCO catalog Nr. 074-2200), which contained 2 g/l NaHCO<sub>3</sub>, through repeated pipetting. According to a modified procedure by De St. Groth and Scheidegger [*J. Immunol. Methods* (1980), 35, 1]  $5 \times 10^7$  cells of the lymph node were fused with  $5 \times 10^7$  PAI mouse myeloma cells (J.W. Stocker et al., *Research Disclosure*, 217, May 1982, 155-157), which were in the process of logarithmic growth. The cells were mixed, collected through centrifugation then resuspended through gentle shaking in 2 ml 50% (v/v) polyethylene glycol in IMEM at room temperature and diluted through slow addition of 10 ml IMEM during 10 minutes of careful shaking. The cells were collected through centrifugation and re-

suspended in 200 ml of the complete medium [IMEM + 20% fetal calves' serum, glutamine (2.0 mM), 2-mercaptoethanol (100  $\mu$ M), 100  $\mu$ M hypoxanthine, 0.4  $\mu$ M aminopterin and 16  $\mu$ M thymidine (HAT)]. The suspension was distributed among 10 tissue culture dishes, each with 96 indentations and, without change of medium, incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> and at relative humidity of 98%, for 11 days.

The antibodies are characterized by their inhibiting effect to TNF binding with HL60 cells or through their binding to antigen during the filter test according to Example 1. The following procedure was employed for detection of biological activity of anti-(TNF-BP)-antibodies :  $5 \times 10^6$  HL60 or U937 cells were incubated in the complete RPMI 1640 medium together with affinity-free monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those that are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10  $\mu$ g/ml. After an hour of incubation at 37°C the cells were collected through centrifugation and washed with 4.5 ml PBS at 0°C. They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2), which contained additional 0.1% sodium azide and <sup>125</sup>I-TNF $\alpha$  (10<sup>6</sup> cpm/ml) with or without addition of unmarked TNF $\alpha$  (see above). The specific radioactivity of <sup>125</sup>I-TNF $\alpha$  amounted to 700 Ci/mmol. The cells were incubated for 2 hours at 4°C, collected and washed four times with 4.5 ml PBS, which contained 1% BSA and 0.001% Triton X 100 (Fluka), at 0°C. The radioactivity linked to the cells was measured in a  $\gamma$ -scintillation counter. In a comparable experiment the radioactivity linked to cells that were not treated with anti-(TNF-BP) antibodies was determined (about 10,000 cpm/ $5 \times 10^6$  cells).

#### Example 4

##### Affinity Chromatography

For further purification a monoclonal anti-(44 kDa TNF-BP) antibody (2.8 mg/ml gel), as obtained according to Example 3, TNF (3.0 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel) were coupled covalent to CNBr-activated Sepharose 4B

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(Pharmacia, Uppsala, Sweden). The cell extract obtained according to Example 2 was fed through the thus established columns which were arranged in series in the following sequence: BSA-Sepharose precolumn, immunoaffinity column [anti-(55 kDa-TNF-BP)-antibody], TNF $\alpha$ -ligand affinity column. After the completed task the two last mentioned columns were separated and individually washed with 100 ml each of the following buffer solutions: (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 E/ml aprotinine; (2) PBS, 0.1% Triton X-100, 0.5 M NaCl, 10 mM benzamidine, 100 E/ml aprotinine; and (3) PBS, 0.1% Triton X-100, 10mM benzamidine, 100 E/ml aprotinine. The immunoaffinity column as well as the TNF $\alpha$ -ligandaffinity column were then eluated, each one separately, with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 E/ml aprotinine. The fractions of each column that were active during the filter test as per Example 1 were always united afterwards and neutralized with 1 M Tris pH 8.0 .

The united TNF-BP active fractions of immunoaffinity chromatography on the one hand and of TNF $\alpha$ - ligandaffinity chromatography on the other were once more applied each to a small TNF $\alpha$ - ligandaffinity column for further purification. Afterwards these two columns were washed each with 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 E/ml aprotinine, (2) PBS, 0.1% Triton X-100, 0.5 M NaCl, 10 mM ATP, 10 mM benzamidine, 100 E/ml aprotinine, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decymatoside .Subsequently the columns were eluated with 100 mM glycine pH 2.5, 100 mM NaCl, 0,2% decylmaltoside. Fractions of 0.5 ml from each column were collected separately and the active fractions per filter test (Example 1) of each column united each separately and concentrated in a Centricon unit (Amicon, Molecular weight exclusion 10'000).

#### Example 5

##### Separation by means of HPLC

The active fractions obtained according to Example 4 were applied with reference to the different origins (immunoaffinity chromatography resp. ligandaffinity chromatography) each separately to C1/C8 reversed-phase HPLC columns (ProRPC, Pharmacia, 5x20 mm), which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octylglucoside. The columns were then eluted with a linear acetonitrile gradient (0-80%) in the same buffer with a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were separately united (detection per Example 1).

#### Example 6

##### Separation by means of SDS-PAGE

The active fractions obtained according to Example 5 and per filter test (Example 1) were further separated according to [34]. For that purpose the probes were heated for three minutes to 95°C in SDS probe buffer and subsequently separated electrophoretically on a 12% acrylamide separation gel with a 5% collecting gel. As reference for determination of the apparent molecular masses on the SDS-PAGE gel the following reference proteins were used: phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), Carboanhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Under the conditions mentioned two bands of 55 kDa and 51 kDa as well as three weaker bands of 38 kDa, 36 kDa and 34 kDa were obtained for probes that had been obtained according to Example 4 through TNF $\alpha$ -ligandaffinity chromatography from immunoaffinity chromatography eluates and had been further separated through HPLC according to Example 5. These bands were transferred electrophoretically in a Mini Trans Blot System (Bio Rad, Richmond, CA, USA) for 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA). Afterwards the PVDF membrane was dyed either with 0.15% ~~Serva-Blau (Serva, Heidelberg, BRD) in methanol/water/glacial acetic acid (50/40/10~~ parts by volume) to protein or blocked with defatted powdered milk and subse-

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quently incubated with  $^{125}\text{I}$ -TNF $\alpha$  according to the filter test conditions described in Example 1 for the detection of bands with TNF-BP activity. It was shown that all the bands exhibited in the protein color specifically bound TNF $\alpha$ . All these bands bound in the Western blot according to Towbin et al. [38] including the monoclonal anti-55 kDa-TNF-BP- antibody produced according to Example 3. In that case a rabbit-anti-mouse -immunoglobulin antibody marked by Na $^{125}\text{I}$  radioactively, affinity-free(mouse immunoglobulin -Sephadose-4B affinity column) according to the process described in Example 1 was employed for autoradiographic detection of this antibody.

Probes, which are obtained according to Example 4 through double TNF $\alpha$  - ligandaffinity chromatography of the flowthrough of immunoaffinity chromatography and are further separated through HPLC according to Example 5, showed two additional bands of 75 kDa and 65 kDa, both of which bound TNF specifically during the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kDa TNF-BP)- antibody produced according to Example 3. They did react, however, with a monoclonal antibody, which had been produced according to Example 3 starting from the 75 kDa band (anti-75 kDa TNF-BP-antibody).

#### Example 7

##### Amino Acid Sequence Analysis

For amino acid sequence analysis the active fractions obtained according to Example 5 and per filter test (Example 1) were separated by means of the SDS-PAGE conditions described in Example 6 and now being reduced (SDS probe buffer with 125 mM dithiothreitol). The same bands were found as per Example 6, but they all showed about 1-2 kDa higher molecular masses in comparison with Example 6 because of the reducing conditions of SDS-PAGE. These bands were then transferred according to Example 6 to PVDF membranes and dyed with 0.15% 35 Serva-Blau in ~~methanol/water/glacial acetic acid (50/40/10 parts per volume) during 1 minute,~~

decolored with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, airdried and then cut out. During all the steps the conditions given by Hunkapiller [34] were followed to avoid N-terminal blockage. At first the purified TNF-BP were placed unchanged into the amino acid sequencing. To obtain additional sequence information, the TNF-BP were cleaved with bromocyanide (Tarr, G.E. in "Methods of Protein Microcharacterization", 165-166 op. cit.), trypsin and/or proteinase K, after reduction and S-carboxymethylation [Jones, B.N. (1986) in "Methods of Microcharacterization", J.E. Shively, ed. Human Press, Clifton N.J. 124-125] and the peptides were separated by means of HPLC according to known methods of protein chemistry. So prepared the probes were then sequenced in an automated gas phase-microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, CA, USA) with an on-line automated HPLC PTH- amino acid analyzer (Applied Biosystems Model 120, ABI see above) behind it, with the following amino acid sequences being determined :

1. For the 55 kDa band (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

and

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys

where X stands for an amino acid residue that could not be identified.

2. For the 51 kDa and the 38 kDa bands (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu

3. For the 65 kDa band (according to non-reducing SDS-PAGE): during N-terminal sequencing of the 65 kDa band two parallel sequences were identified without interruption down to the 15th residue. Since one of the sequences corresponded to a partial sequence of ubiquitin [36,37] the following sequence was derived for the 65 kDa band:

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Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys.

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where X stands for an amino acid residue that could not be identified.

Additional peptide sequences for 75(65)kDa-TNF-BP were identified:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-LeuLeu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu

and

Val-Phe-Cys-Thr

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala

and

Leu-Cys-Ala-Pro

and

Val-Pro-His-Leu-Pro-Ala-Asp

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

Where X stands for an amino acid residue that could not be identified.

#### Example 8

##### Analysis of Base Sequences of Complementary DNA (cDNA)

Starting with the amino acid sequence according to formula AI, complete degenerated oligonucleotides corresponding to amino acid residues 2-7 and 17-23 were, with consideration of the genetic code, synthesized in suitable complementarity ("sense" and "antisense" oligonucleotide). Total cellular RNA was isolated from HL60 cells [42,43], and the first strand of cDNA was synthesized through oligo-dT-priming or through priming with the "antisense" oligonucleotide by means of a cDNA synthesis kit (RPN 1256 Amersham, Amersham England) according to the manufacturer's instructions. This cDNA strand and the two synthesized degenerated "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction

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(PCR, Perkin Elmer Cetus, Norwalk, CT, USA, according to manufacturer's instruction) to synthesize the base sequence encoded for the amino acid residues 8-16 (formula IA) as a cDNA fragment. The base sequence of this cDNA fragment is as follows: 5'-AGGGAGAAGAGAGATAGTGTGTGTC-3'. This cDNA fragment was used as probe to identify a cDNA clone encoded for the 55 kDa TNF-BP in a  $\lambda$ gt 11-cDNA-gene bank of human placenta (42,43) through known procedures. This clone was then cut from the  $\lambda$ -vector with the usual methods and cloned into the M13mp18/M13mp19 bacteriophages (Pharmacia, Uppsala, Sweden) (42,43). The nucleotide sequence of this cDNA clone was determined by means of a sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to information from the manufacturer. The nucleotide sequence and derived from it the amino acid sequence for the 55 kDa TNF-BP and its signal peptide (amino acid "-28" to amino acid "0") is illustrated in Fig.1 with abbreviations for bases and amino acids customary at the present state of technology. From sequence comparisons with other already known receptor protein sequences about 180 N-terminals containing amino acids as well as 220 C-terminal domains containing amino acid may be identified, which are separated by a transmembrane region of 19 amino acids (underlined in Fig.1) typical according to sequence comparisons. Hypothetical glycosylation locations are marked with stars above the corresponding amino acid in Fig.1 .

Analog technologies were employed basically for identification of partial cDNA sequences encoded for 75/65 kDa TNF-BP with, in this case, genomic human DNA and from peptide IIA derived completely degenerated 14-mer (polymer ..Transl.) and 15-mer "sense and "antisense" nucleotides being used, to produce a primary, 26 bp cDNA probe in a polymerase chain reaction. This cDNA probe was then used for identification of cDNA clones of various lengths in a HL-60 CDNA library. This cDNA library was produced by means of isolated HL60 RNA and a cDNA cloning kit (Amersham) per instructions of the manufacturer. The sequence of such a cDNA clone is illustrated in Fig.4, with repeated sequencing leading to the

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following correction . In place of the serine in position 3 a threonine must be put which is encoded by "ACC" and not by "TCC".

#### Example 9

##### Expression in COS 1-Cells

For the expression in COS cells vectors were constructed which originated from plasmid "pN11". The plasmid "pN11" contains the efficient promoter and enhancer of the "major immediate-early " gene of the human cytomegalovirus ("HCMV"; Boshart et al., Cell 41 , 521-530, 1985). Behind the promoter there is a short DNA sequence, containing several restriction interfaces that occur only once in the plasmid ("polylinker"), among them the interfaces for HindIII, Ball, BamHI and PvuII (see sequence).

PvuII

5'- AAGCTTGGCCAGGATCCAGCTGACTGACTGATCGCGAGATC-3'

3'- TTCGAACCGGTCTAGGTCGACTGACTGACTAGCGCTCTAG-5'

Behind these interfaces there are three translation stop codons in all three reading rasters . Behind the polylinker sequence is the second intron and the polyadenylation signal for the preproinsulin gene of the rat (Lomedico et al. Cell 18, 545-558, 1979). The plasmid contains furthermore the replication origin of the SV40 virus as well as a fragment from pBR322, which confers ampicillin resistance on E. coli bacteria and makes possible the replication of the plasmid in E. coli .

For construction of the expression vector "pN123" this plasmid "pN11" was cut with the restriction endonuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was then isolated from an agarose gel (V1). The 5' overhanging nucleotides of the EcoRI cut 1.3 kb fragments of the 55 kDa TNF-BP=cDNA (see Example 8) were filled with the help of

Klenow enzyme. Subsequently this fragment was isolated from an agarose gel (F1).

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Afterwards V1 and F1 were united by means of T4 ligase. *E. coli* HB 101 cells were then transformed with this ligation batch according to familiar methods [42]. With the aid of restriction analyses and DNS sequencing according to familiar methods [42] transformants were identified, which had been transformed with a plasmid that contained the 1.3kb *EcoRI* fragment of the 55 kDa TNF-BP-cDNA in correct orientation for the expression via the HCMV promoter. This vector received the designation "pN123".

The following procedure was used for construction of the vector "pK19". A DNA fragment, which contains only the cDNA coded for the extracellular part of the 55 kDa TNF-BP (amino acids -28 to 182 according to Fig.1) was obtained by means of PCR technology (Saiki et al., *Science* 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used for amplification of the cDNA encoded for the extracellular part of 55 kDa TNF-BP from "pN123".

BAMHI

5'-CACAGGGATCCATAGCTGTCTGGCATGGGCTCTCCAC-3'

ASP718

3'-CGTGACTCCTGAGTCCGTGGTGTATTATCTCTAGACCATGGCCC-5'

By means of these nucleotides two stop codons of the translation were also introduced behind amino acid 182. The thus amplified fragment was cut with BamHI and Asp718 and the resulting projecting ends were filled with the aid of the Klenow enzyme; this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the whole batch used for transformation of *E. coli* HB101, as already described. Transformants which were transformed with a plasmid that contained the DNA fragment in correct orientation for expression via the HCMV-promoter, were identified by means of DNA sequencing (see above). The plasmid isolated from it received the designation "pK19".

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Transfection of the COS cells with plasmids "pN123" or "pK19" was carried out according to the lipofection method published by Felgner et al. (*Proc. Natl.*

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Acad. Sci. USA 84, 7413-7417, 1987). 72 hours after the completed transfection the cells transfected with "pN123" were analyzed according to known methods with  $^{125}\text{I}$ -TNF $\alpha$  for binding. The result of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of binding data obtained in that way (Fig.2A) is illustrated in Fig. 2B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For that purpose PVC microtitre plates (Dynatech, Arlington, VA, USA) with 100  $\mu$ /hole of a rabbit-anti-mouse immunoglobulin (10  $\mu$ g/ml PBS) were sensitized. The plate was subsequently washed and incubated (3 hours, 20°C) with an anti-55 kDa TNF-BP antibody, which was detected according to Example 3 through its antigen binding and isolated, but does not inhibit TNF binding to cells. The plate was then washed again and incubated overnight at 4°C with 100  $\mu$ /hole of the culture supernatants (diluted 1:4 with buffer A containing 1% defatted milk powder : 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na-azide). The plate was emptied and incubated with buffer A (10<sup>6</sup> cpm/ml, 100  $\mu$ /hole) containing  $^{125}\text{I}$ -TNF $\alpha$  with or without addition of 2  $\mu$ g/ml unmarked TNF for two hours at 4°C. Afterwards the plate was washed four times with PBS, the individual holes were cut out and measured in a  $\gamma$ -counter. The results of five parallel transfections (columns # 2,3,4,6 and 7), of two control transfections with the pN11 vector (columns #1,5) and of a control with HL60-cell lysate (column #8) are illustrated in Fig. 3.

#### Example 10

##### Expression in Insect Cells

For expression in a baculovirus expression system a start was made with the plasmid "pVL941" (Luckow and Sommers, 1989, "High Level Expression of Nonfused Foreign Genes with Autographa California Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39), which was modified as follows. The sole EcoRI-interface in "pVL941" was removed by cutting the plasmid with EcoRI and filling

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the protruding 5'ends with Klenow enzyme. The thus obtained plasmid pVL941/E- was digested with BamHI and Asp718 and the vector rump subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

	BamHI	EcoRI	Asp718	
5'	-	GATCCAGAATTCATAATAG	-	3'
3'	-	GTCTTAAGTATTATCCATG	-	5'

E. coli HB101 was transformed with the ligation batch and transformants, which contained a plasmid in which the oligonucleotide was correctly inserted, were identified through restriction analysis and DNA sequencing according to known methods (see above); this plasmid was called "PNR704". For construction of the transfer vector "pN113" this plasmid "pNR704" was cut with EcoRI, treated with alkaline phosphatase and the thus generated vector rump (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of 55 kDa TNF-BP-cDNA, cut with EcoRI like above, was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid that contained the cDNA insert in the correct orientation for expression via the polyhedrin promoter, were identified (see above). The vector isolated from it received the designation "PN113".

The following steps were taken for construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI-fragment of the 55 kDa TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BstI and ligated with the following synthetic oligonucleotide :

	BstI	Asp718
5'	-	GCACCACATAATAGAGATCTGGTACCGGGAA - 3'
3'	-	GTGTATTATCTCTCTAGACCATGGCCC - 5'

By means of the above adapter two stop codons of the translation behind amino acid 182 and an interface for the restrictions endonuclease Asp718 are

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inserted. After completed ligation the batch was digested with EcoRI and Asp718 and the partial 55 kDa TNF-BP fragment (F3) was isolated. Furthermore plasmid "pNR704", which was also cut with Asp718 and EcoRI, was ligated with F3 and the ligation batch transformed in *E. coli* HB101. Identification of the transformants, which contained a plasmid in which the partial 55 kDa TNF-BP cDNA was integrated correctly for the expression, was carried out as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was followed in construction of the transfer vector "pN124". The cDNA fragment described in Example 9, which encodes for the extra-cellular part of 55 kDa TNF-BP, was amplified with the oligonucleotides mentioned by means of PCR technology, as described in Example 9. This fragment was cut with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cut with BamHI and Asp718 and the vector rump (V4) was isolated (see above). The fragments V4 and F4 were ligated, used to transform *E. coli* HB101 and the recombinant transfer vector "pN124" was identified and isolated, as described.

The following procedure was used for transfection of insect cells. 3 µg of the transfer vector "pN113" were transfected with 1 µg DNA of Autographa californica-nuclear-polyhedrosis virus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedrin negative viruses were identified and purified from "Plaques" [52]. With these recombinant viruses Sf9 cells were again infected as described in [52]. After 3 days in the culture the infected cells were examined for binding of TNF with  $^{125}\text{I}$ -TNF $\alpha$ . For that purpose the transfected cells were washed with a Pasteur pipette from the cell culture dish and, with a cell density of  $5 \times 10^6$  cells /ml culture medium [52] containing 10 ng/ml  $^{125}\text{I}$ -TNF $\alpha$ , as well as in the presense and absence of 5 µg/ml unmarked TNF $\alpha$ , they were resuspended and incubated on ice for two hours. Afterwards the cells were washed with a clean culture medium and the cellbound radioactivity was counted with a  $\gamma$ -counter (see

---

Table 2].

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Table 2

Cells	Cellbound radioactivity/ $10^6$ cells
Non-infected cells (control)	60 cpm
Infected cells	$1600 \pm 330$ cpm <sup>1)</sup>

<sup>1)</sup> Average value and std. deviation from 4 experiments

Example 11

Analogous to the method described in Example 9 the cDNA fragment encoded for the extracellular range of the 55 kDa TNF-BP was now amplified in a polymerase chain reaction with the following oligonucleotides as primers :

Oligonucleotide 1 :

Sst I

5' - TAC GAG CTC GGC CAT AGC TGT CTG GCA TG - 3'

Oligonucleotide 2 :

Sst I

5' - ATA GAG CTC TGT GGT GCC TGA GTC CTC AG -3'

This cDNA fragment was ligated into the pCD4-H $\gamma$ 3-vector [DSM 5523; European Patent Application Nr. 90107393.2; Japanese Patent Application Nr. 108967/90; US Patent Application Ser. No. 510773/90], from which the CF4-cDNA had been removed via the Sst I-restriction interfaces. Sst interfaces are present in vector pCD4-H 3 before, inside and behind the CD4 partial sequence section. The construct was transfected by means of protoplast fusion according to Oi et al. (Proc. Nat. Acad. Sci. USA 80 , 825-829, 1983) in J558-myeloma cells (ATCC Nr. TIB6). Transfectants were



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selected into the basic medium (Dulbecco's modified Eagle's medium, 10% fetal calves' serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol) by addition of 5  $\mu\text{g/ml}$  myphenolic acid and 250  $\mu\text{g/ml}$  xanthine (Traunecker et al., Eur. J. Immunol. 16, 851-854 [1986]). The expression product secreted by the transfected cells could be purified through the usual methods of protein chemistry, for instance TNF-8P antibody affinitychromatography. Where not already specifically mentioned, standard methods like for example that of Freshney, R.I. in "Culture of Animal Cells", Alan R. Liss, Inc. New York (1983) were used for growing the cell lines used, for cloning, selecting, resp. for expansion of the cloned cells.

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### Claims

1. Non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF in homogeneous form, as well as their physiologically compatible salts.
2. Compounds according to Claim 1, which are characterized by molecular masses according to SDS-PAGE under nonreducing conditions of about 55 kDa and 75 kDa.
3. Compounds according to one of the Claims 1 and 2, which contain at least one

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile;  
Ser-Thr-Pro-Glu-Lys-Glu-GlyGlu-Leu-Glu-Gly-Thr-Thr-Thr-Lys;  
Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys;  
Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Glu-Pro-Leu-Glu;  
Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu;  
Val-Phe-Cys-Thr;  
Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala;  
Leu-Cys-Ala-Pro;  
Val-Pro-His-Leu-Pro-Ala-Asp;  
Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

where X stands for an unidentified acid residue.

4. TNF sequences which are encoded for non-soluble proteins or soluble as well as non-soluble fragments thereof, that bind TNF, with such DNS sequences to be selected from the following:

- (a) DNA sequences, as shown in Fig.1 or Fig.4, as well as their complementary strands, or those that these sequences include;
- (b) DNA sequences, which hybridize with sequences as defined under (a) or with their fragments.
- (c) DNA sequences, which do not hybridize with sequences as defined under (a) and (b), because of degeneracy of the genetic code, but which are encoded for polypeptides with exactly the same amino acid sequence.

5. DNA sequences according to Claim 4, which include a combination of two partial DNA sequences, with one partial sequence encoded for soluble fragments of non-soluble proteins that bind TNF, and the other partial sequence encoded for all domains save the first of the constant region of the heavy chain of human immunoglobulins, like IgG, IgA, IgM resp. IgE.

6. DNA sequences according to Claim 5, with said human immunoglobulins being of

the IgM type resp. those of class IgG.

7. DNA sequences according to Claim 6, where said human immunoglobulins are those of type Ig1 resp. Ig3.

8. Recombinant proteins from DNA sequences encoded according to one of the Claims 4-7, such as allelic variants, or deletion analogs, substitution analogs or addition analogs thereof.

9. Vectors, which contain DNA sequences according to one of the Claims 4-7 and are suitable for expression of proteins encoded by these DNA sequences in prokaryotic as well as eukaryotic host systems.

10. Prokaryotic as well as eukaryotic host systems which have been transformed with a vector according to Claim 9.

11. Host systems according to Claim 10, which are mammalian or insect cells.

12. Antibodies directed against a compound according to one of the Claims 1-3 or 8.

13. A method for isolation of a compound according to one of the Claims 1-3, characterized in that the following purification steps are carried out sequentially: production of a cell extract, immunoaffinity chromatography and/or simple or multiple ligandaffinity chromatography, HPLC and preparative SDS-PAGE and if so desired cleavage of the compounds thus isolated and/or transference into suitable salts.

14. A method for production of a compound per Claim 8, which is characterized in that a transformed host system according to Claim 10 or 11 is cultivated in a suitable medium and in that such compounds are isolated from the host system proper or from the medium.

15. Pharmaceutical preparations, characterized in that they contain one or more compounds according to one of the Claims 1-3 or 8, if so desired in combination with additional pharmaceutically effective substances and/or non-toxic, inert, therapeutically compatible carrier materials.

16. Pharmaceutical preparations for treatment of diseases, in which TNF is involved, ~~where such preparations are characterized in that they contain one or more compounds~~

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according to one of the Claims 1-3 or 8, if so desired in combination with additional pharmaceutically effective substances and/or non-toxic, inert, therapeutically compatible carrier materials.

17. Use of a compound according to one of the Claims 1-3 or 8 for treatment of diseases.

18. Use of a compound according to one of the Claims 1-3 or 8 for treatment of diseases, in which TNF is involved.

19. A compound as claimed in one of the Claims 1-3 or 8 whenever it has been produced by a method as claimed in Claims 13 or 14.

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Figur 1

Fig.1

```

-185 GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
-125 CCTCACTGTCACCCCAAGGCACTTGGGACGTCTGGACAGACCGAGTCCCCGGAAGCC
-65 CCAGCACTGCCGCTGCCCACTGCCCTGAGCCCAATGGGGGAGTGAGAGGCCATAGCTG
-28.
-30 MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
-5 TCTGGCATGGGCTCTCCACGCTGCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
+1
-10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
55 TTGGTGGGAATATACCCCTCAGGGGTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG
***
10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
115 AAGAGAGATAGTGTGTGTCCCAAGGAAATATATCCACCCTCAAATAATTGATTTC
30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
175 TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGAGGAT
50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
235 ACGGACTGCAGGGAGTGTGAGAGCGGTCCTTCACCGCTTCAGAAAACCACCTCAGACAC
70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
295 TGCCTCAGCTGCTCCAAATGCCAAAGGAAATGGGTGAGGTGGAGATCTCTTCTGCACA
90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCTGACCGGCATTATTGGAGTGAA
***
110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGACCTCTCCTGC
130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475 CAGGAGAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535 TGTGTCTCCTGTAGTAACTGTAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCAG
170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595 ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGTGTGCCCTGGTCAAT
190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655 TTCTTTGGTCTTTGCCCTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG
210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715 TGGAGTCCAAGCTCTACTCCATTGTTTGTGGGAATCGACACCTGAAAAGAGGGGGAG
***
230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775 CTTGAGGAACTACTACTAAGCCCTGGCCCCAAACCAAGCTTCAGTCCCACTCCAGGC
250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
835 TTCACCCCAACCTGGGCTTCAGTCCCGTGCCAGTTCCACCTTCACCTCCAGCTCCACC
270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
895 TATACCCCGGTGACTGTCCCACTTTGCGGCTCCCGCAGAGAGGTGGCACCACCCTAT
290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
955 CAGGGGCTGACCCCATCCTTGGCAGACCCCTGCCTCCGACCCCATCCCCAACCCCTT

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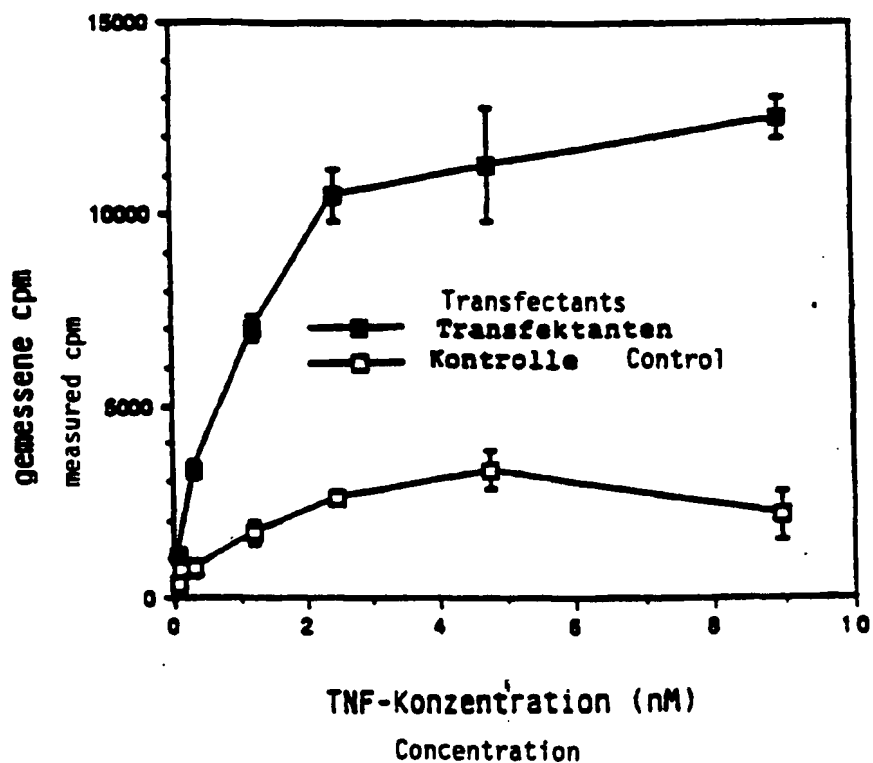
Figur 1 (Forts.) Fig.1 (cont.)

310 GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr  
 1015 CAGAAGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG  
  
 330 LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu  
 1075 CTGTACGCCCTGGTGGAGAACGTGCCCCGTTGCGCTGGAAGGAATCGTGCGGCGCCTA  
  
 350 GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu  
 1135 GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGGCGCTGCCTGCGCGAG  
  
 370 AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu  
 1195 GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCAGCGCGGGGCGGAGGCGACGCTG  
  
 390 GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu  
 1255 GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG  
  
 410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg  
 1315 GAGGCGCTTTGCGGCCCGCGCGCCCTCCCGCCCGCGCCAGTCTTCTCAGATGAGGCTGC  
 1375 GCGCCTGCGGGCAGCTCTAAGGACCGTCTGCGAGATCGGCTTCCAACCCCACTTTTTTC  
 1435 TGGAAAGGAGGGGTCTGCGAGGGGCAAGCAGGAGCTAGCAGCGCGCTACTTGGTGCTAAC  
 1495 CCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCGCGCGACAGTCAGCGCTGTGGG  
 1555 CCGGAGAGAGAGGTGCGCGGTGGGCTCAAGAGCCTGAGTGGGTGGTTTTCGAGGATGAGGG  
 1615 ACGCTATGCCTCATGCGCGTTTGGGTGTCTCACCAGCAAGGCTGCTCGGGGGCCCTG  
 1675 GTTCGTCCCTGAGCCTTTTTACAGTGCAAGCAGTTTTTTTTGTTTTGTTTTGTTTT  
 1735 GTTTGTTTTTAAATCAATCATGTTACACTAATAGAACTTGGCACTCCTGTGCCCTCTG  
 1795 CCTGGACAAGCACATAGCAAGCTGAAGTGTCTAAGGCAGGGGCGAGCACGGAACAATGG  
 1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACTAAAATTCTGAAGTTAAAAAAA  
 1915 AACCCGAATTC



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Figur 2A    Fig.2a



Figur 2B

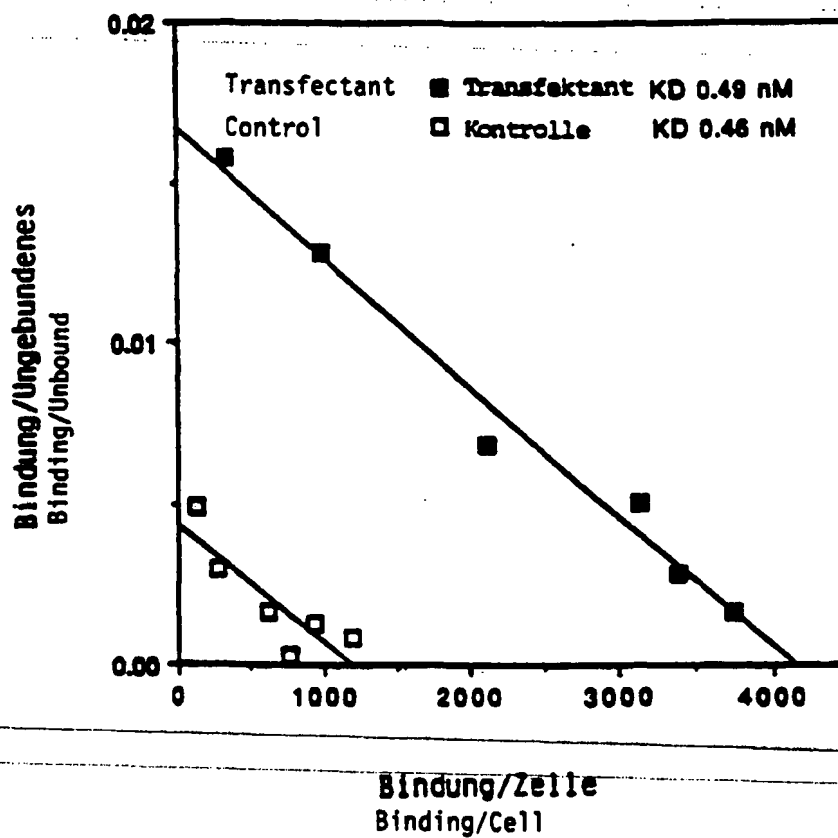


Figure 3 Fig.3

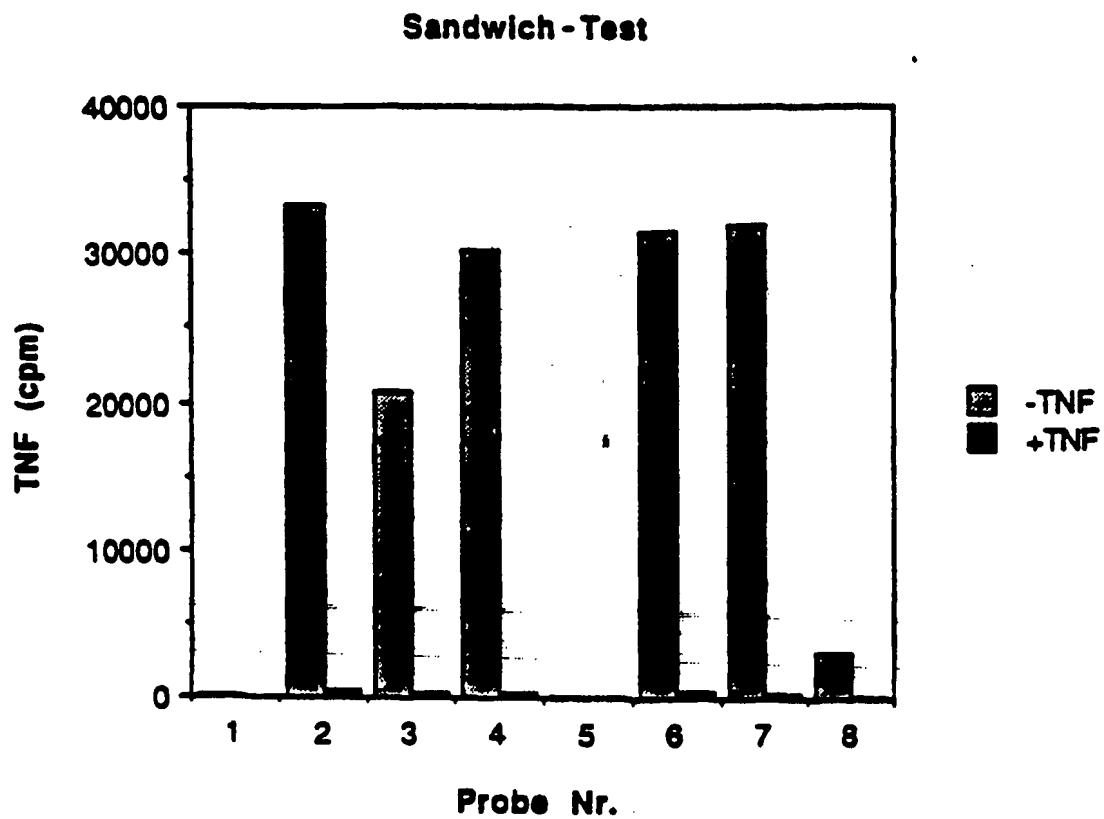


Figure 4 Fig.4

1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal  
 1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAAGTGGGTT  
 21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys  
 61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAACTCAAGCCTGC  
 41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTyrTyrCysAlaLeuSerLys  
 121 ACTCGGGAACGAAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG  
 61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla  
 181 CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCCGGGCTTCGGCGTGGCC  
 81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer  
 241 AGACCAGGAAGTGAACATCAGACGTGGTGTGCAGGCCCTGTGCCCCGGGGACGTTCTCC  
 101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle  
 301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACAGATCTGTACGTTGGTGGCCATC  
 121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla  
 361 CCTGGGAATGCAGCAGGGATGCAGTCTGCACGTCCACGTCCCCACCCGGAGTATGGCC  
 141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer  
 421 CCAGGGGCGAGTACACTTACCCAGCCAGTGTCCACAGATCCCAACACACGCAGCCAGT  
 161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro  
 481 CCAGAACCCAGCACTGCTCCAGCACCTCCTTCTGCTCCCAATGGGCCCCAGCCCCCA  
 181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla  
 541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC  
 201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys  
 601 TTGGGTCTACTAATAATAGGAGTGGTGAAGTGTGTCATCATGACCCAGGTGAAAAGAG  
 221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly  
 661 CCCTTGTCCTGCAGAGAGAGCCAGGTGCCTCACTTGCTGCGGATAGGCCCGGGGT  
 241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer  
 721 ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC  
 261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla  
 781 CTGGAGAGCTCGCCAGTGCCTTGGACAGAGGGGCGCCCACTCGGAACCAOCCACAGGCA

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